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APPLICATION NOTE

# icIEF Analysis of Adeno-Associated Virus (AAV) Proteins for Gene Therapy


## INTRODUCTION

In medicine, gene therapy is the process where nucleic acids are delivered to a patient's cells as a therapeutic drug to treat genetic diseases including hematological, immunological, neurodegenerative, and metabolic disorders, as well as several types of cancers. Once in the nucleus, the therapeutic DNA or RNA replaces a mutant gene with a functional gene, knocks-out a mutated gene that is functioning incorrectly, or introduces a new gene into the body to help fight disease. Moreover, the development of versatile gene-editing technologies like CRISPR, which make it faster and more reliable to modify target DNA, elevates the promise gene therapy holds as a potential treatment option.

All gene therapies utilize either viral or non-viral vectors to deliver the DNA or RNA into the host cell. Viral vectors infect the host cell to introduce the genetic material and are more efficient at transfecting the host cell compared to non-viral vectors like cationic lipids or chemical carriers. However, they can have immunogenic side effects depending on the specific virus used. Therefore, the choice of appropriate viral vector is a critical component when developing the drug. Adeno-associated virus (AAV) is non-pathogenic, and thus incurs only minimal immune response, making it an ideal gene therapy vector.

As with all therapeutic drugs, product characterization is of utmost importance in order to ensure drug safety and stability. For gene therapies, this includes characterizing the delivery vector before the drug is packaged. In this study, we demonstrate how imaged-capillary isoelectric focusing (icIEF) can be used to characterize the charge heterogeneity of AAV vectors to ensure product stability and identity. Maurice delivers this critical analysis as an automated platform that removes the variability typically encountered with platforms that require more hands-on time, and generates high-resolution data in less than 10 minutes. More importantly, Maurice offers 3-5x higher sensitivity than absorbance with the native fluorescence detection mode, offering significant advantages when characterizing low concentration AAV samples.

## MATERIAL AND METHODS

### REAGENTS

AAV2 ($1x10^{13}$ GC/mL) and AAV6 (5 lots that ranged from $3.8\ x10^{12}$ – $8.5\ x\ 10^{13}$ GC/mL) samples were obtained from Vigene Biosciences. The a la carte pI marker 9.46 was acquired from ProteinSimple (catalog #102349) while the SimpleSol Protein Solubilizer, methylcellulose, Pharmalyte 3-10 and 5-8, and the Maurice 5.85 and 8.40 pI markers were all obtained from the ProteinSimple Maurice cIEF Method Development Kit (catalog #PS-MDK01-C). Dithiothreitol (DTT) from TOCRIS (catalog #3154) was reconstituted using HPLC grade deionized water to a stock concentration of 80 mM. Dimethyl sulfoxide (DMSO) was purchased from Sigma (catalog #D2650-5x5ML) along with Formamide (catalog #F9037).

### INTACT AAV ANALYSIS

AAV samples were analyzed with a method that does not disassociate assembled capsids, leaving them intact during focusing on Maurice. To prevent aggregation, the AAV samples were diluted 25-fold into a master mix, with the final prepared sample containing 50% SimpleSol, 0.35% methylcellulose, 4% 3–10 Pharmalyte, and Maurice pI markers 5.85 and 9.46. The intact AAV samples were run using a Maurice cIEF cartridge (catalog #PS-MC02-C) and pre-focused for 1 minute at 1,500 V then focused at 3,000 V for 7 minutes. Absorbance and native fluorescence images (20- and 80-seconds) were captured and analyzed using Compass for iCE software.

## DENATURED AAV ANALYSIS

The AAV samples were denatured by heating the sample in the presence of 33% DMSO and 16.5 mM DTT for 10 minutes at 70 °C, then cooled to room temperature. Samples were then prepared for Maurice analysis by diluting the denatured sample 5-fold, with the final prepared sample containing 0.35% methylcellulose, 2% Pharmalyte 5–8 and 2% Pharmalyte 3–10, 40% formamide, and Maurice pI standards 5.85 and 8.40. Samples were separated using a Maurice cIEF cartridge (catalog #PS-MC02-C) for 1 minute at 1,500 V followed by 12 minutes at 3,000 V. Absorbance and native fluorescence images (20- and 80-seconds) were captured and analyzed using Compass for iCE software.

# REPRODUCIBLE ANALYSIS OF INTACT AAV

We developed a method to analyze intact AAV particles on Maurice using native fluorescence to get a better understanding of the product as it pertains to particle stability and identity. We first assessed the intra-assay reproducibility of the intact AAV method on Maurice to determine whether Maurice could be used to reliably quantitate AAV samples.

When intact AAV2 and intact AAV6 (~3 x $10^{12}$ GC/mL) samples were examined on Maurice using absorbance and native fluorescence detection, a similar apparent pI for both serotypes around pI ~9.0 was observed (**Figure 1**). AAV2 appeared as a single peak, just below pI ~9.0 while the intact AAV6 sample resolved into 2 peaks and had an apparent pI ~9.25. A clear advantage of using fluorescence detection was observed as native fluorescence is 3–5x more sensitive compared to absorbance detection, which translates to higher signal-to-noise ratios. For example, the signal-to-noise for the AAV2 peak just below pI ~9.0 was 13.9 using absorbance detection and 36.2 with an 80-second exposure using native fluorescence detection. For AAV6, the peak using absorbance detection was very close to the baseline with a signal-to-noise of 2.9, bringing into question whether the peak can be reliably detected using absorbance. In comparison, the peak using native fluorescence using an 80-second exposure had a signal-to-noise of 24.1. This gain in sensitivity made it possible to save precious AAV sample since less starting material was required for analysis.

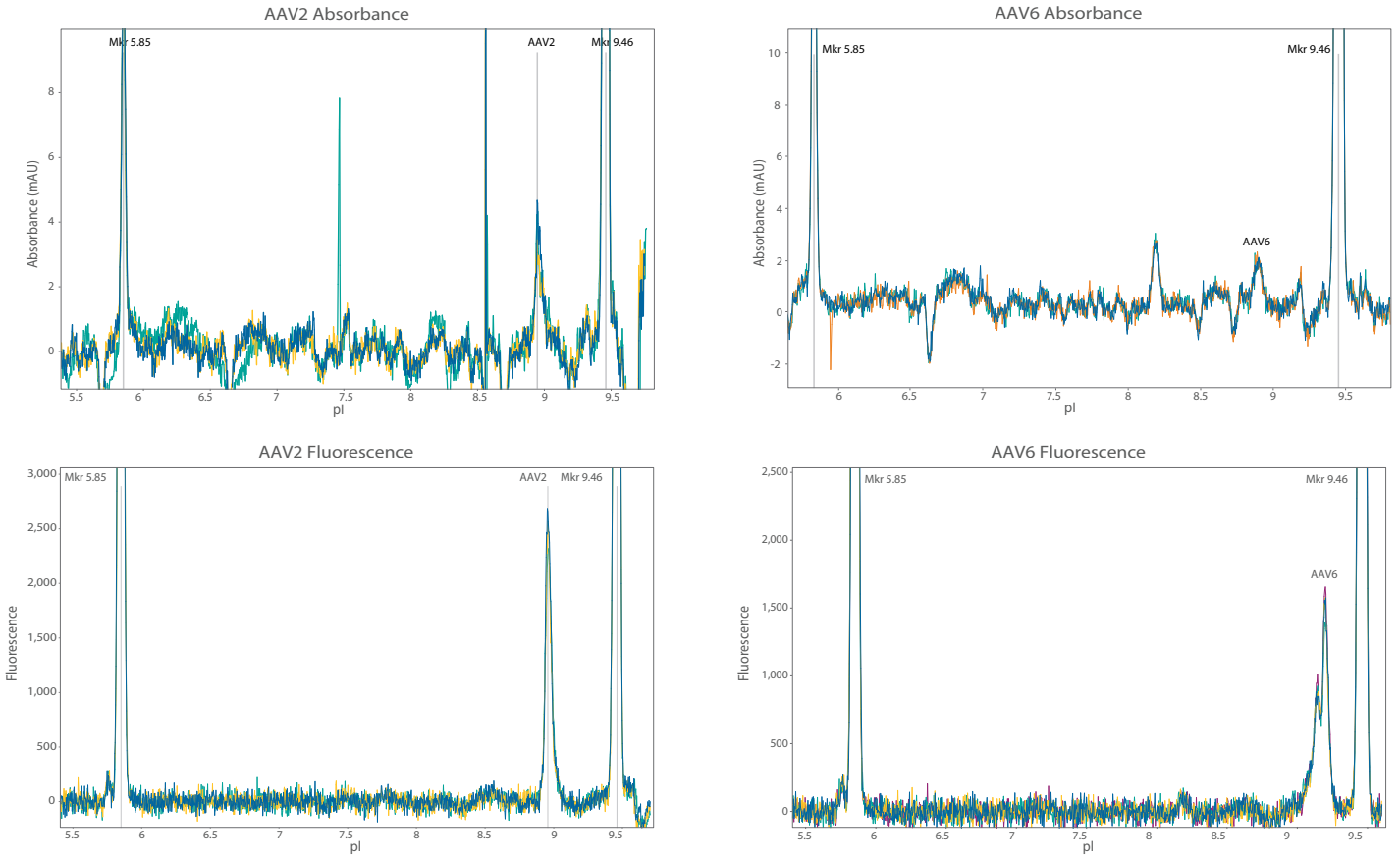


**FIGURE 1. Apparent pI comparison of intact AAV2 and AAV6 particles.** The intact AAV2 resolves as a single peak just below pI ~9.0 (left) while the intact AAV6 resolves as 2 peaks at an apparent pI ~9.25 (right). Native fluorescence detection (bottom) was clearly more sensitive compared to absorbance detection (top), making it possible to save precious sample when performing intact AAV analytics.

The intra-assay reproducibility of the intact method was then gauged by analyzing the total area for quadruplicate injections of the AAV2 and AAV6 samples (**Figure 2**). Results for both viral samples were very reproducible, with a %RSD of 3.95% and 4.30% for AAV2 and AAV6, respectively (**Table 1**).

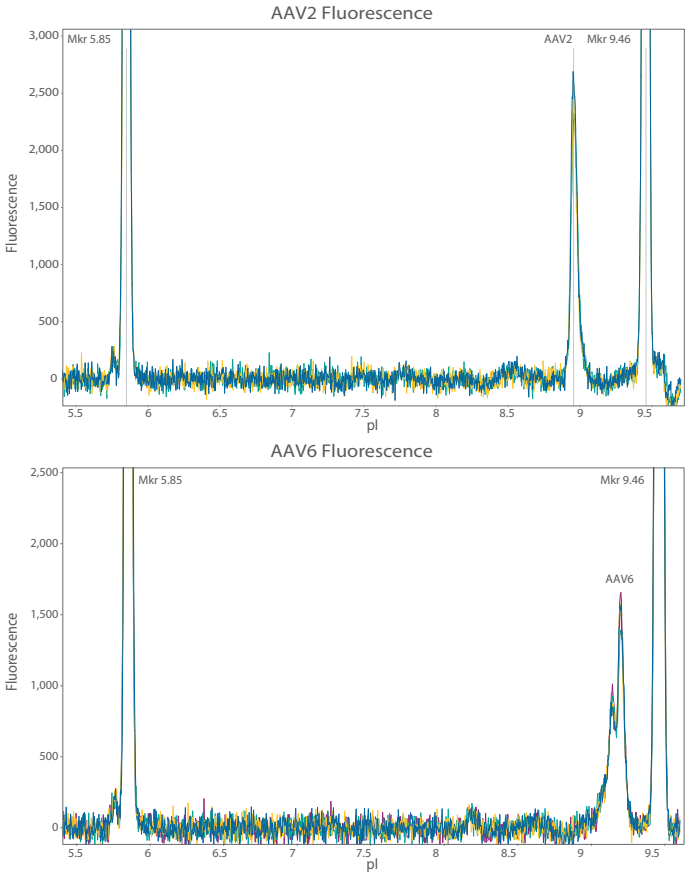


FIGURE 2. An overlay of quadruplicate injections of intact AAV2 (top) and AAV6 (bottom) visually demonstrates the intra-assay reproducibility of the intact AAV method. Fluorescence data are shown with 80-second exposures.

| SAMPLE | INTACT AAV2 AREA | INTACT AAV6 AREA |
|---|---|---|
| Injection 1 | 47867 | 34711 |
| Injection 2 | 43590 | 36932 |
| Injection 3 | 47992 | 34636 |
| Injection 4 | 46225 | 38318 |
| Mean | 1827.69 | 36149.25 |
| %RSD | 3.95 | 4.30 |

TABLE 1. Quantitative results from the quadruplicate injections of intact AAV2 and AAV6 demonstrates the intra-assay reproducibility of the intact AAV method. The results were very reproducible with %RSDs of 3.95% and 4.30% for the intact AAV2 and AAV6, respectively.

We then assessed the inter-assay reproducibility by running an AAV6 sample (~ 3 x $10^{12}$ GC/mL) in triplicate on three separate days, for a total of nine injections. Due to limited sample availability, only the intact AAV6 was used to evaluate the inter-assay reproducibility of the method (**Figure 3**). Quantitation of the total peak area suggests the method was highly reproducible, with a %RSDs of 6.6% (**Table 2**).

## REPRODUCIBLE ANALYSIS OF DENATURED AAV

AAV viral proteins are subject to several post-translational modifications, including glycosylation and deamidation. Stress-induced deamidation of viral proteins can lead to a loss of vector activity, capsid assembly, and transduction efficiency[1].

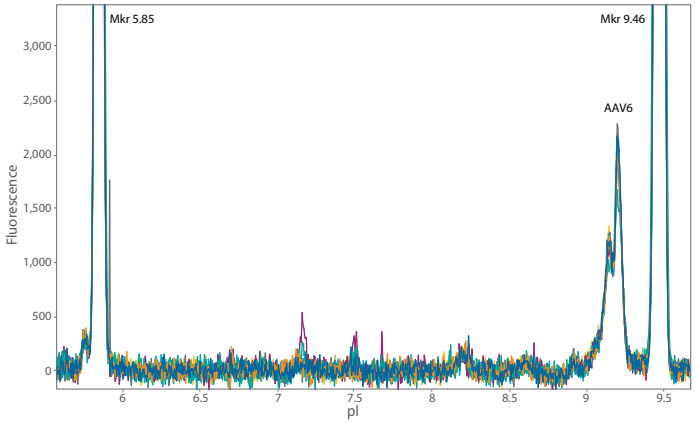


FIGURE 3. An overlay of nine injections, run over the course of three days, of intact AAV6 visually demonstrates the inter-assay reproducibility of the intact AAV method (data shown for 80 second fluorescence exposure).

| | TOTAL PEAK AREA |
|---|---|
| Day 1 | 42245<br>49481<br>50006 |
| Day 2 | 47217<br>48165<br>45943 |
| Day 3 | 51304<br>51270<br>53871 |
| Mean | 48834 |
| %RSD | 6.60 |

TABLE 2. Quantitative results from nine injections, where samples were run in triplicate over three days, confirm the inter-assay reproducibility of the intact AAV method, with a %RSD of 6.60% for the total peak area.

Denatured icIEF methods are commonly utilized to monitor charge heterogeneity of monoclonal antibodies induced by sialylation, glycation, and deamidation, so we hypothesized similar assessments could be performed on AAV viral proteins.

To confirm that Maurice can be used to characterize the viral vector, we denatured AAV2 (~1 x $10^{12}$ GC/mL) prior to analysis and evaluated the reproducibility of the denatured AAV method. AAV2 charge variants were clearly resolved and reproducible when evaluated visually using an overlay of triplicate electropherograms using both absorbance and native fluorescence detection (**Figure 4**). Quantitation of the total peak area further confirms the intra-assay reproducibility of the data, as the % RSDs for the total area was 3.2% using absorbance detection and 1.5% using native fluorescence detection (**Table 3**). As with the intact AAV method, native fluorescence detection was more sensitive compared to absorbance detection, which translates to better baseline resolution and higher signal-to-noise ratios. A 10x increase was observed when assessing the minor peak at pI 6.5, as the signal-to-noise for absorbance and native fluorescence with an 80-second exposure was 2.1 and 23.6, respectively. Again, this results in significant sample savings, as less AAV starting material is required to perform icIEF analysis using fluorescence detection on Maurice.

The inter-assay reproducibility of denatured methods was also assessed using the AAV6 sample. Te denatured AAV6 (~6 x $10^{12}$ GC/mL) was run in triplicate on three different days. The inter-assay performance was highly reproducible (**Figure 5**). The data quantitation confirmed this, as the peaks with greater than 10% average percent composition all had %RSDs under 7.5% for percent peak area, and the standard deviation for the pI values were all under 0.02 (**Table 4**).

## MAURICE icIEF METHOD IS SENSITIVE AND LINEAR

To establish the method's sensitivity, we then serially titrated the denatured AAV2 from ~3 x $10^{12}$ GC/mL down to ~3 x $10^{11}$ GC/mL (**Figure 6**). A blank buffer was also included as a negative control. Maurice was able to detect AAV2 protein from a sample with as little as 3 x $10^{11}$ GC/mL using native fluorescence detection, with a calculated limit of detection of ~1 x $10^{11}$ GC/mL. A strong linear correlation was observed across this titration range, with an $R^2$ of 0.9956.

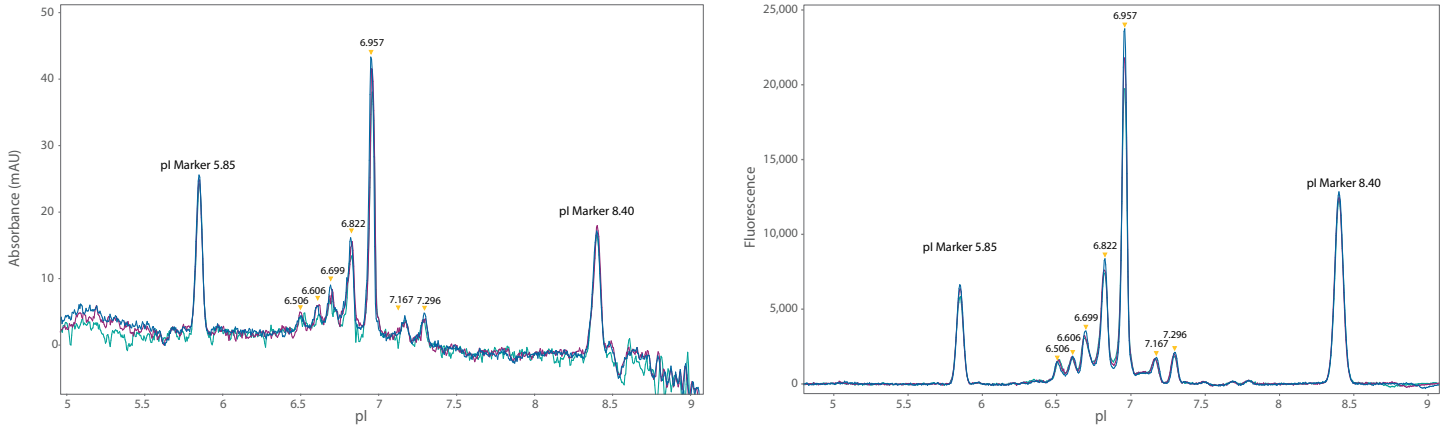


**FIGURE 4. The denatured icIEF method for AAV2 is reproducible for both absorbance (left) and native fluorescence (right) detection within a run.** Shown are overlays of three injections for both detection modes. Data generated with native fluorescence detection had improved signal-to-noise ratios and baseline resolution compared to absorbance detection.

| INJECTION | TOTAL PEAK AREA (ABSORBANCE) | TOTAL PEAK AREA (NATIVE FLUORESCENCE) |
|---|---|---|
| 1 | 926 | 488620 |
| 2 | 999 | 474643 |
| 3 | 985 | 491055 |
| Mean | 970 | 484773 |
| %RSD | 3.2 | 1.5 |

**TABLE 3. Denatured AAV2 quantitation on Maurice is reproducible. %RSDs for the total peak area were 3.2% using UV absorbance detection and 1.5% using native fluorescence detection.**

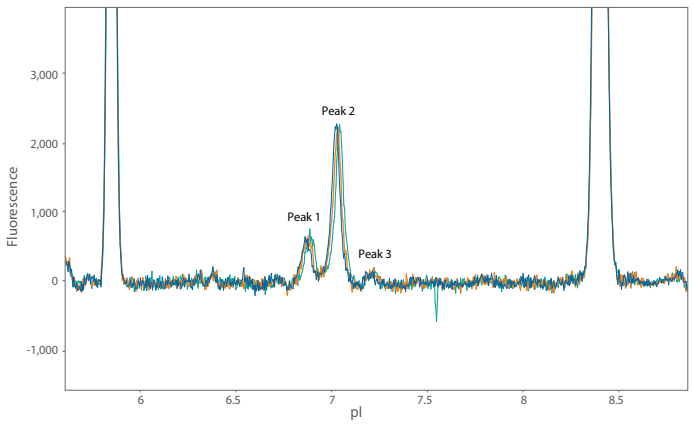


FIGURE 5. An overlay of three injections of denatured AAV6, run over the course of three days, visually demonstrates the inter-assay reproducibility of the denatured icIEF method.

| | PEAK % | | | | PEAK PI | | |
|---|---|---|---|---|---|---|---|
| | Peak 1 | Peak 2 | Peak 3 | | Peak 1 | Peak 2 | Peak 3 |
| Day 1 | 22.7<br>22.7<br>22.6 | 75.3<br>74.7<br>73.0 | 2.0<br>2.6<br>4.4 | Day 1 | 6.86<br>6.87<br>6.89 | 7.03<br>7.03<br>7.04 | 7.21<br>7.22<br>7.22 |
| Day 2 | 23.5<br>23.0<br>24.2 | 74.9<br>74.9<br>73.5 | 1.6<br>2.1<br>2.3 | Day 2 | 6.86<br>6.87<br>6.91 | 7.03<br>7.04<br>7.04 | 7.19<br>7.22<br>7.21 |
| Day 3 | 19.7<br>19.4<br>20.2 | 77.3<br>78.5<br>78.0 | 3.0<br>2.0<br>1.8 | Day 3 | 6.91<br>6.89<br>6.89 | 7.05<br>7.05<br>7.06 | 7.23<br>7.24<br>7.27 |
| Mean | 22.0 | 75.6 | 2.4 | STD | 0.02 | 0.01 | 0.02 |
| %RSD | 7.5 | 2.4 | 33.2 | | | | |

TABLE 4. Quantitative results from the nine injections of denatured AAV6, where triplicate injections were run on three different days, demonstrates the inter-assay reproducibility of the denatured AAV icIEF method. The results were very reproducible, as pI value standard deviation was all under 0.02 and %RSDs for percent peak area for peaks with greater than 10% composition were all under 7.5%.

# USE MAURICE TO MONITOR AAV PARTICLE STABILITY

Temperature was used to stress-test intact or denatured AAV2 and AAV6 samples to determine whether Maurice could be used to monitor viral vector stability or capsid protein deamidation. We first evaluated the stress-tested intact AAV protein. A sample of AAV6 was treated at either 37 °C over the weekend, 50 °C for 10 minutes, or 60 °C for 10 minutes, and then compared to the reference AAV6 sample on Maurice using the intact method (**Figure 7**). The AAV6 particles were quite stable, remaining intact over the weekend at 37 °C or at 50 °C for 10 minutes. However, the AAV6 particle was not stable when it was stressed at 60 °C for 10 minutes, as we did not observe a peak signature at the same pI of the reference sample. AAV particles have been previously shown to have serotype-specific melting temperatures that range from 60–90 °C[2]. These data suggest the intact method for icIEF can be used to potentially monitor particle stability.

We then stressed denatured samples to determine whether Maurice could be used to monitor the stability of denatured viral samples. Denatured AAV2 and AAV6 samples were split into two aliquots – one aliquot was stressed at 95 °C for 5 minutes while the other aliquot was kept fresh on ice to be used as a reference sample. The stressed and reference samples were then analyzed on Maurice. A profile change was clearly observed with both AAV2 and AAV6 samples that were stressed with temperature, as there was an increase in acidic species, indicating icIEF analysis with Maurice can be used to monitor whether the sample has started to change (**Figure 8**). Quantitation of the peak area percent also indicates that the stressed sample has changed from the reference sample (**Table 5**) and, therefore, may have negative efficacy and safety implications if it's used as a delivery mechanism for gene therapy.

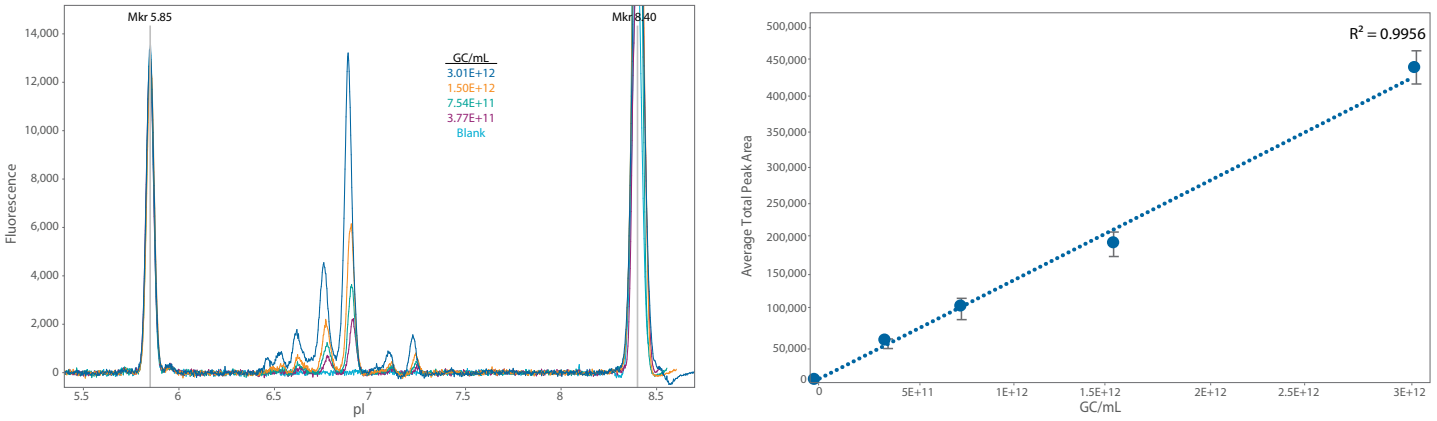


FIGURE 6. The denatured iciEF method for AAV2 is sensitive and shows good linearity. AAV2 was titrated from ~3 x $10^{12}$ down to 3 x $10^{11}$ GC/mL (top) and was detected with as little as 3 x $10^{11}$ GC/mL AAV2 using native fluorescence detection. Strong linearity was also observed (bottom) across the titration range tested with an $R^2$ of 0.9956.

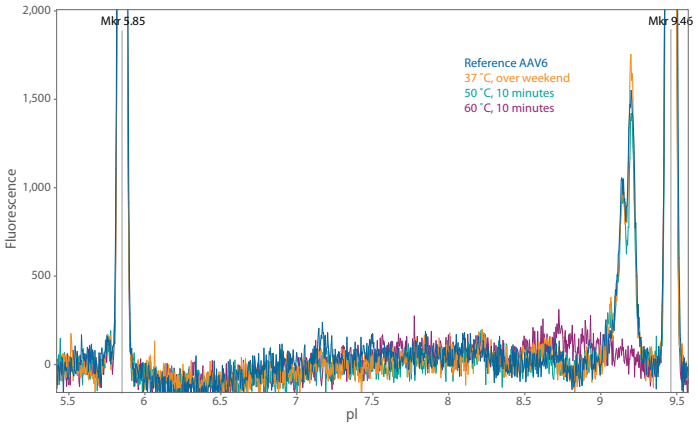


**FIGURE 7. Intact AAV6 analysis following incubation at various temperatures.** After 10 minutes at 60 °C, the AAV6 particle cannot be observed and is no longer stable. The change in peak signature after stress-testing indicates Maurice can be used to monitor viral vector stability.

# MAURICE MONITORS AAV LOT-TO-LOT VARIATION

Finally, we evaluated whether Maurice icIEF native fluorescence could be used to monitor the lot-to-lot variation for identity in the viral vector source material. We obtained five different lots of AAV6, each with a different genomic content per mL (GC/mL) content and analyzed them with the intact icIEF methods on Maurice using native fluorescence (**Figure 9**). All five lots generated similar peak profiles at around pI ~9.25; however, an additional peak at a lower pI was observed in one lot, indicating the sample is different from the other four lots.

The total peak area for each lot was then graphed with the reported GC/mL to determine whether the Maurice AAV intact method shows a correlation between genomic content and protein composition (**Figure 10**). When the data were compared to the reported GC/mL from the vendor, the lots with the lower total peak area generally were the lots with lower GC/mL content.

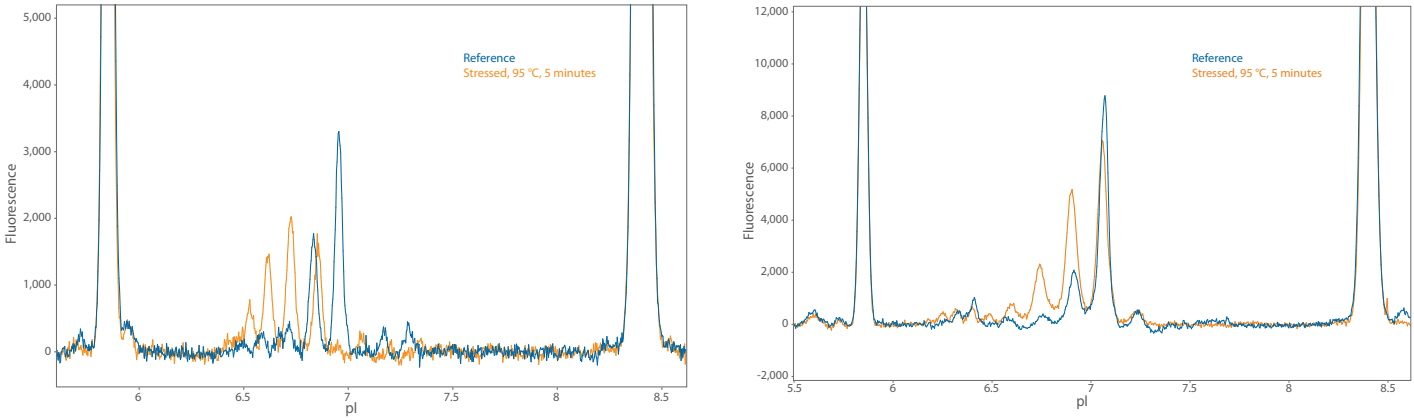


**FIGURE 8. The stress-tested denatured AAV2 (left) and AAV6 (right) samples generated peak profiles that were significantly different compared to the reference sample using Maurice icIEF native fluorescence detection, indicating the stress-tested sample has changed.** This indicates Maurice can be used to monitor the stability of denatured viral vector samples.

| | AAV2 % PEAK AREA | |
|---|---|---|
| Peak pI | Reference Sample | Stressed Sample |
| 6.53 | ND | 11.8 |
| 6.59 | 4.1 | 24.3 |
| 6.72 | 7.1 | 35.4 |
| 6.83 | 28.5 | 25.2 |
| 6.95 | 50.5 | 2.7 |
| 7.17 | 3.8 | 0.6 |
| 7.29 | 6.0 | ND |

| | AAV6 % PEAK AREA | |
|---|---|---|
| Peak pI | Reference Sample | Stressed Sample |
| 6.60 | ND | 4.7 |
| 6.74 | ND | 15.7 |
| 6.90 | 21.8 | 35.4 |
| 7.06 | 72.9 | 41.2 |
| 7.23 | 5.3 | 3.0 |

TABLE 5. Quantitation of % peak areas for the profiles generated when stressed and reference AAV2 (left) and AAV6 (right) samples were run on Maurice icIEF using native fluorescence detection support the conclusion that the stressing the sample at 95 °C for 5 minutes has changed the sample.

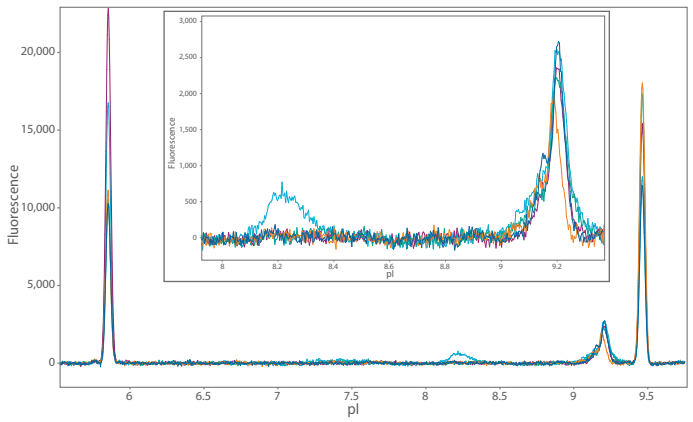


**FIGURE 9. AAV6 lot-to-lot comparison.** Five different lots of AAV6 were analyzed using the intact method. All lots produced a similar profile at pI ~ 9.25, but only one lot had an additional peak at a lower pI.

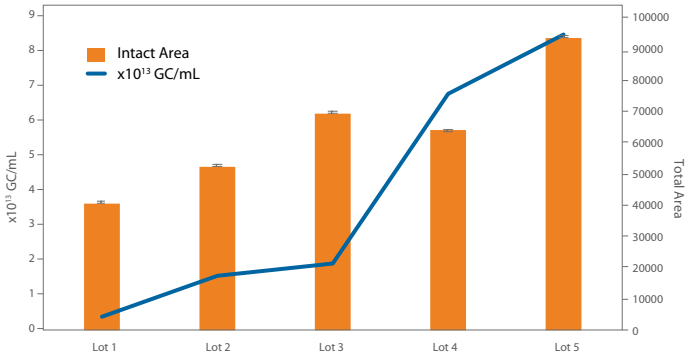


**FIGURE 10. Comparison of AAV6 genomic copy and intact protein area.** Each lot of AAV6 possessed a different genomic copy (GC/mL) number that ranged from 3.8 x $10^{12}$ – 8.53x$10^{13}$ GC/mL, which was plotted against the intact AAV6 total peak area.

## CONCLUSION

Charge heterogeneity is a critical quality attribute required by the FDA for every protein therapeutic to ensure drug safety and efficacy. The Maurice icIEF method delivers this analysis with a fully automated workflow that provides unprecedented reproducibility and sensitivity. This makes it an ideal system to characterize AAV vector stability for gene therapies. In this study, we demonstrated how Maurice can resolve either intact or denatured AAV2 and AAV6 isoforms using absorbance or native fluorescence detection. The data generated with native fluorescence, however, affords data with higher signal-to-noise ratios due to its improved sensitivity compared to absorbance detection, suggesting it should be the preferred detection mode when analyzing viral vector samples on Maurice.

Intra- and inter-assay quantitation using the total peak area was reproducible for both intact and denatured methods using fluorescence detection. The intra- and inter-assay total peak area RSDs were under 4.4% and 6.7%, respectively, for the intact method while intra- and inter-assay RSDs using either total peak area or % peak area were all under 7.6%. The quantitation was also highly linear with an $R^2$ of 0.9956 when denatured AAV2 was titrated from ~3 x $10^{12}$ GC/mL down to ~3 x$10^{11}$ GC/mL. We also subjected AAV2 and AAV6 samples to high temperature for accelerated stress tests to evaluate whether Maurice could be used to monitor vector stability. A change in peak profile was clearly observed in the stressed AAV2 and AAV6 sample when compared to the reference sample, using either the intact or denatured AAV method, indicating that charge heterogeneity analysis using Maurice can be used to monitor AAV stability. Finally, a comparison of five different lots of AAV material with different genomic content demonstrates that the Maurice intact AAV method can be used to monitor lot-to-lot variation between vector source material and inform on a lot's identity and protein content.

Maurice is a powerful platform that can be used to characterize your AAV viral vector to assess AAV lot-to-lot variability, concentration, and stability using stressed samples during the formulation phase of drug development. This means you'll always have the assurance that you're packaging your therapeutic RNA and DNA in a viral vector that will safely and efficiently deliver your drug treatment to the patient.

## REFERENCES

1. Deamidation of amino acids on the surface of adeno-associated virus capsids leads to charge heterogeneity and altered vector function, AR Giles, JJ Sims, KB Turner, L Govindasamy, MR Alvira, M Lock, JM Wilson, *Mol Ther*, 2018; 26(12):2848-2862.

2. Thermal stability as a determinant of AAV serotype identity, A Bennett, S Patel, M Mietzsch, A Jose, B Lins-Austin, JC Yu, B Bothner, R McKenna, M Agbandje-McKenna, *Mol Ther Methods Clin Dev*, 2017; 6:171-182.

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